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ScienceDirect
Comparative Biochemistry and Physiology, Part B 149 (2008) 126–147
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www.elsevier.com/locate/cbpb

Molecular characterization and sex-specific tissue expression of estrogen receptor α (*esr1*), estrogen receptor βa (*esr2a*) and ovarian aromatase (*cyp19a1a*) in yellow perch (*Perca flavescens*)

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Received 3 May 2007; received in revised form 31 August 2007; accepted 1 September 2007
Available online 7 September 2007

Abstract

Yellow perch (*Perca flavescens*) exhibits an estrogen-stimulated sexual size dimorphism (SSD) wherein females grow faster and larger than males. To aid in the examination of this phenomenon, the cDNA sequences encoding estrogen receptor-α (*esr1*), estrogen receptor-βa (*esr2a*) and ovarian aromatase (*cyp19a1a*) for the teleost yellow perch were obtained. Several tissues were analyzed from both male and female adult yellow perch for sex-specific tissue expression. The full length cDNAs of yellow perch *esr1*, *esr2a* and *cyp19a1a* consist of 3052 bp, 2462 bp and 1859 bp with open reading frames encoding putative proteins of 576 amino acids, 555 amino acids and 518 amino acids, respectively. *Esr1* and *esr2a* expression was highest in female ovary and liver tissues with low to moderate expression in other tissues. *Esr2a* showed a more global tissue expression pattern than *esr1*, particularly in males but also in females. *Cyp19a1a* expression was highest in both male and female spleen tissue and oocytes with moderate expression in male pituitary and gill tissue. *Cyp19a1a* expression was moderately high in female liver tissue with undetectable expression in male liver tissue, suggesting its involvement in sexually dimorphic growth. These sequences are valuable molecular tools that can be used in future studies investigating estrogen mechanisms and actions, such as SSD, in yellow perch.

Keywords: Cloning; Fish; Hormone; Phylogenetic tree; Steroid

1. Introduction

Steroid hormone receptors are members of a large family of ligand-activated nuclear transcription factors that are critical to the reproduction, differentiation and development of vertebrates. All steroid receptors are believed to have derived from an ancient estrogen receptor through the process of two large-scale genome expansions, one before the advent of the jawed vertebrates and the second after (Thorton, 2001). Estrogen receptors are members of a family of nuclear transcription factors including receptors for sex steroids, thyroid hormone, retinoids as well as many "orphan" receptors for which no ligands have yet been identified (Hewitt and Korach, 2002). All members of this family share a modular structure that consists of a variable trans-activation domain (A/B), a highly conserved DNA binding domain (C or DBD), a variable hinge region (D), a well-conserved ligand binding domain (E or LBD), and a variable C-terminal region (F) (Hewitt and Korach, 2002; Hawkins and Thomas, 2004). It was believed that there was only one type of estrogen receptor (initially called ERα, but now called *esr1* Sprague et al., 2006) in vertebrates until the discovery of a second estrogen receptor subtype (initially called ERβ, now called *esr2*) in 1996 (Kuiper et al., 1996). The recent discovery of a second *esr2* form in fish (Hawkins et al., 2000) generated designations for the two subtypes as ERβa and ERβb, ERβ1 and ERβ2, or ERγ and ERβ (Ma et al., 2000; Halm et al., 2004; Hawkins et al., 2005). This study utilizes the zebrafish official nomenclature (zfin.org) (Sprague et al., 2003; Sprague et al., 2006) which designates the two fish paralogs as

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doi:10.1016/j.cbpb.2007.09.001

esr2a and *esr2b*. Phylogenetic analyses indicate that the two *esr2* paralogs arose from the duplication of an ancestral *esr2* gene early in the teleost lineage after the split of the tetrapods and fish (Hawkins et al., 2000; Pinto et al., 2006). *Esr1* and the *esr2* orthologs are the products of separate genes (Kuiper et al., 1996) and have distinct, yet partially overlapping, distribution in estrogen target tissues (Kuiper et al., 1997; Xia et al., 2000; Choi and Habibi, 2003) with different ligand binding affinities reported in both mammals (Kuiper et al., 1997) and fish (Hawkins and Thomas, 2004).

In teleosts, the presence of estrogen receptors in specific tissues can be of paramount importance for main events during sexual development, sexual maturation or reproduction (Cavaco et al., 1998). Different fish species have shown different sex-specific tissue expression patterns for estrogen receptors. For example, in adult European sea bass (*Dicentrarchus labrax*), females had strikingly higher ERα expression in liver and pituitary than males, but there were few differences between males and females in either ERβ expression levels (Halm et al., 2004). In adult gilthead seabream (*Sparus auratus*), males showed high expression of all three ERs in testis tissue while females only showed high expression of ERβa in ovary tissue (Pinto et al., 2006). In goldfish (*Carassius auratus*), both males and females showed the highest expression levels of ERα and ERβ2 in pituitary tissue, while the highest levels of ERβ1 were in gonad tissue (Choi and Habibi, 2003). Largemouth bass (*Micropterus salmoides*) females had significantly higher levels of ERα in liver tissue while ovary tissue showed the highest levels of expression for both ERβs (Sabo-Attwood et al., 2004). These types of comparative sex-specific tissue expression analyses have been important in identifying target tissues of estrogen in each species.

The primary physiological ligand for estrogen receptors is the sex steroid 17β-estradiol or simply estradiol. Estradiol (E_2), the most physiologically active form of estrogen, is synthesized mainly in the ovary by p450 aromatase ($p450_{arom}$, cyp19a), a cyp19 gene product, which is a member of the cytochrome *P-450* superfamily. Aromatase is the terminal steroidogenic enzyme in the biosynthesis pathway of estrogen by catalyzing the formation of aromatic C_{18} estrogen from C_{19} androgen (Simpson et al., 1994). Teleosts are unique from other vertebrates in that two structurally and functionally different cyp19a1 isoforms, deriving from two separate loci (Kazeto et al., 2001; Chang et al., 2005), have been identified and are designated *cyp19a1a* and *cyp19a1b* (Sprague et al., 2006). In bony fish, these forms are preferentially expressed in the ovary (*cyp19a1a*) and brain (*cyp19a1b*), which is generally characterized by exceptionally high levels of aromatase activity (Callard et al., 1990). A phylogenetic analysis shows that the *cyp19a1b* forms share higher homology between species than with the *cyp19a1a* form in the same species (Blázquez and Piferrer, 2004).

Few studies have investigated sex-specific tissue expression levels (excluding gonads) of *cyp19a1a,* but in mixed sex studies *cyp19a1a* has been shown to have a very global expression pattern (Sawyer et al., 2006). *Cyp19a1a* is primarily expressed in the ovary but also shows considerable expression in other tissues, most notably brain and spleen (Kobayashi et al., 2004). For example, adult southern flounder (*Paralichthys lethostigma*) showed highest expression of *cyp19a1a* in ovary and spleen tissues with much lower levels present in brain, testis, gill and liver and no expression detected in muscle, heart, intestine or kidney (Luckenbach et al., 2005). Conversely, Atlantic halibut (*Hippoglossus hippoglossus*) adults showed *cyp19a1a* expression in brain, heart, gonad, pituitary and spleen tissues with no expression in gill, intestine or liver tissues (van Nes et al., 2005). And, in a recent study, Atlantic halibut males showed similar *cyp19a1a* expression levels to females in brain, pituitary, gill, and spleen tissues however males had substantially higher kidney, stomach and intestine expression than females (Matsuoka et al., 2006). These studies highlight the variation in sex-specific tissue expression of *cyp19a1a* in fish and further underscore the need for additional research in this area.

The yellow perch (*Perca flavescens*) is one in a group of important fishes which exhibit a sexual dimorphism (SSD) wherein females grow faster than males. Studies have identified 17β-estradiol (E_2) as a growth stimulator in yellow perch SSD (Malison et al., 1985; Malison et al., 1986) but the growth promoting effects of E_2 were only noticeable in fish of 80–110 mm total length (TL) or greater (Malison et al., 1985). This critical size range is also the specific minimum body size for the onset of vitellogenesis and spermatogenesis in females and males, respectively (Malison et al., 1986). This is also the period when females normally begin to outgrow males (Schott et al., 1978) and a female-biased SSD begins to be manifested, pointing towards an upregulation of estrogen receptors on target tissues (ovary, liver or pituitary) and a coinciding increase in tissue expression of growth factors. These studies point out a clear linkage of growth and reproductive development in this species and suggest the presence of an intricate, sex-specific regulatory relationship between growth factors and E_2. In an effort to gain more understanding into the yellow perch estrogen-stimulated SSD, the cDNAs for genes involved with the somatotropic axis (growth hormone (GH) Roberts et al., 2004; prolactin (PRL), somatolactin (SL) and insulin-like growth factor-I (IGF-I) Lynn and Shepherd, 2007) were recently cloned and characterized. As a compliment to those studies, and as a precursor to future work on SDD in yellow perch, the full length cDNAs for *esr1*, *esr2a*, and *cyp19a1a* were cloned and sequenced and sex-specific tissue expression of these genes was investigated in this study. Additionally, the deduced amino acid sequences translated from the yellow perch cDNA sequences were used to generate alignments and phylogenetic trees with all known teleost sequences providing a comprehensive analysis of the phylogenetic relationships for each individual gene.

2. Methods

2.1. Cloning and sequence analyses

Gravid ovaries were collected from adult female yellow perch (*P. flavescens*) brought into the Panuzzo Fish Co. (Lorain, OH, USA) by recreational fishermen for cleaning. Tissues were

Table 1
Forward (F) and reverse (R) primer sequences used to generate initial PCR products for *esr1*, *esr2* and *cyp19a1a*

Gene		Start	Primer sequence
esr1	F	652	GCT ACG AAG TGG GCA TGA TGA AAG GA
	R	1545	TCT CCA GCA GCA GGT CGT ACA GAG G
esr2	F	1074	CGT CTG GTC GTG TGA GGG GTG TAA G
	R	1832	AGG CAC ATG TTG GAG TTG AGG AGG A
cyp19a1a	F	358	AGT ACG GAG ACA TTG TCA GAG TCT
	R	1531	TTG TTG GTC TGT GGG AGG CAG TC

collected from live fish, immediately frozen on dry ice, transported back to the University of Kentucky and stored at −80 °C until total RNA was extracted with the GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). RNA samples were treated with amplification grade DNase I (Sigma) and quantified on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was generated with ligated 5′ and 3′ anchor primers from 5 μg total RNA using the GeneRacer™ Kit (Invitrogen, Carlsbad, CA, USA).

GenBank was searched for neoteleost *esr1*, *esr2*, and *cyp19a1a* cDNAs and several sequences were aligned using Vector NTI Suite v. 7.0 (Informax, Inc., Frederick, MD) and GeneDoc (Nicholas et al., 1997). Consensus sequences, or fragments thereof, were used with Primer3, a web-based primer design program (Rozen and Skaletsky, 2000), and generic neoteleost primers for genes of interest were generated. Generated primers (Table 1) were used with first-strand cDNA from gravid ovary tissue to obtain initial internal PCR products. The PCR protocol used was identical to the one described in Lynn and Shepherd (2007). PCR products were electrophoresed, excised, purified, ligated into a vector and transformed into competent cells as described in Lynn and Shepherd (2007). The plasmid DNA was then extracted from the bacterial cells using the GenElute™ Plasmid Miniprep Kit (Sigma), quantified and used in a PCR reaction with BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Products were sequenced at the University of Kentucky Advanced Genetic Technologies Center.

Species-specific *esr1*, *esr2a* and *cyp19a1a* primers were developed based on the sequences generated and the PCR, cloning, and sequencing procedure was repeated as necessary (with 5′ and 3′ GeneRacer™ primers) to achieve full length (5′ UTR+CDS+3′UTR) sequences. BLASTN v. 2.2.14 (Altschul et al., 1997) searches were used to determine similarities with other teleosts and to verify gene identity. To amplify the full coding region of yellow perch *esr1*, *esr2a* and *cyp19a1a* cDNAs, new primers were designed (Table 2). Total RNA (750 ng) extracted from gravid ovary tissue was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA) to generate cDNA templates for PCR cloning. Again, the PCR protocol used was identical, except with longer (120 s) extension steps, to the one described in Lynn and Shepherd (2007). PCR products were electrophoresed, excised, purified, cloned and sequenced as described above to verify sequence data and primer-gene specificity. GeneDoc (Nicholas et al., 1997) was used to generate amino acid sequence alignments of *esr1*, *esr2a*, and *cyp19a1a* from teleost sequences given by a BLASTP v. 2.2.14 search (Altschul et al., 1997). Percent identities of yellow perch proteins with other proteins revealed by the BLASTP search were determined using DNASTAR Lasergene MegAlign v. 7.2.0. Alignments were produced in Clustal X1.81 (Thompson et al., 1994) and used to generate phylogenetic tree data using the Neighbor Joining tree method (Saitou and Nei, 1987) with 1000 bootstrap trials and TreeView v. 1.61 was used to create a visual phylogenetic tree.

2.2. Sex-specific tissue expression

The tissues used in this study were the exact same tissues used in Lynn and Shepherd (2007) which were collected from adult yellow perch kept in approved aquaculture facilities at the University of Kentucky, Lexington, KY (USA) (IACUC #00251L2001). Three male and three female yellow perch were sampled in spring (March) and euthanized with MS 222 before tissues were harvested. Brain, pituitary, gill, heart, liver, stomach, spleen, kidney, skeletal muscle and gonad tissues were collected from both males and females and ovaries were stripped of oocytes which were also kept for analysis. From these tissue samples, total RNA was extracted, treated with amplification grade DNase, quantified and reverse transcribed to cDNA as described in Lynn and Shepherd (2007). Primers listed in Table 2 were used in a PCR reaction as described in Lynn and Shepherd (2007), with the exception of a longer (120 s) extension step, to determine sex-specific tissue expression of *esr1*, *esr2a* and *cyp19a1a* mRNA. Following PCR amplification, products were electrophoresed in 1% agarose gels and visualized using ethidium bromide staining. cDNA template quality and quantity (equal loading of material) were verified by analyzing for β-actin mRNA levels using real-time quantitative PCR (qPCR) (data not shown).

3. Results

3.1. Esr1

Yellow perch *esr1* cDNA (Accession DQ984124) was found to consist of 3052 bp containing an open reading frame (ORF)

Table 2
Forward (F) and reverse (R) primer sequences used to generate full length cDNA coding region PCR products for *esr1*, *esr2a*, and *cyp19a1a*

Gene		Start	Primer sequence	Size (bp)
esr1	F	19	CAC CGT GTG CCC TCT CCA GTG AC	1781
	R	1800	CTT CAC ATG TTG CTT GCC GTG CTC	
esr2a	F	521	CGA TAC TGA CAC CGT CTG TAG TTG C	1718
	R	2239	GCT GTG GTC CAC CTC CAG AGT GCT	
cyp19a1a	F	27	GTT TGT GCA GTC GTG TGC AGG TTG T	1605
	R	1632	CAG AGT CTC AGA GTT TTT GCC AGC TTC C	

of 1731 bp, a 41 bp 5′-UTR and a 1280 bp 3′-UTR. The open reading frame encoded a protein of 576 amino acids consisting of 5 domains common to estrogen receptors (Fig. 1). The isoelectric point and molecular mass of the *esr1* peptide, as calculated by Vector NTI BioPlot, are 8.62 and 63,386.52 Da, respectively. Comparison of the deduced amino acid sequence of the yellow perch *esr1* with other known teleost ERαs is shown in Fig. 2, with percent identity (listed in the last row of the figure) determined using DNASTAR Lasergene v. 7.2.0. Yellow perch *esr1* was also determined to have percent identities with the following vertebrate ERαs: 51.3% American alligator and spectacled caiman; 51.1% Nile crocodile; 50.7%

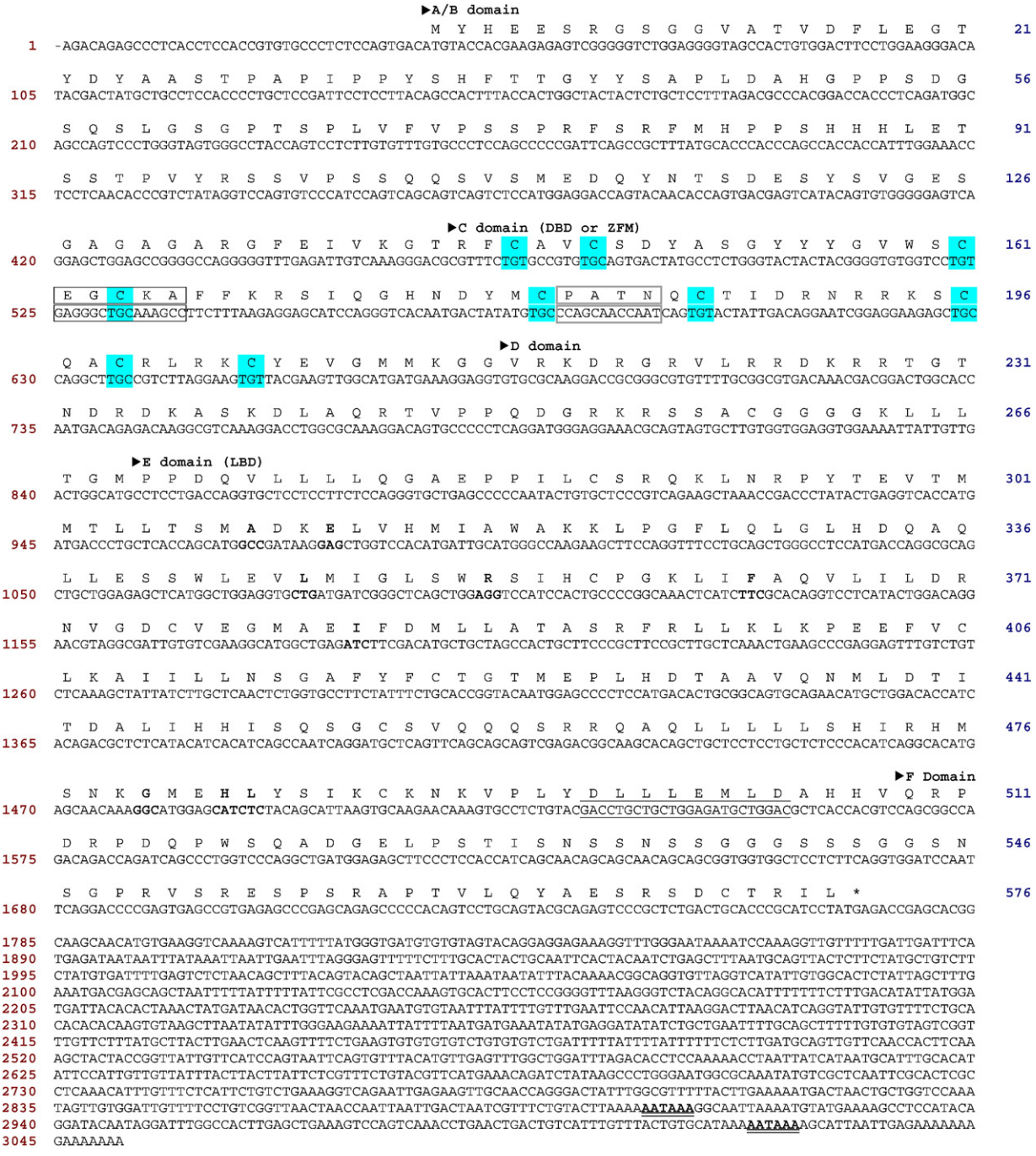
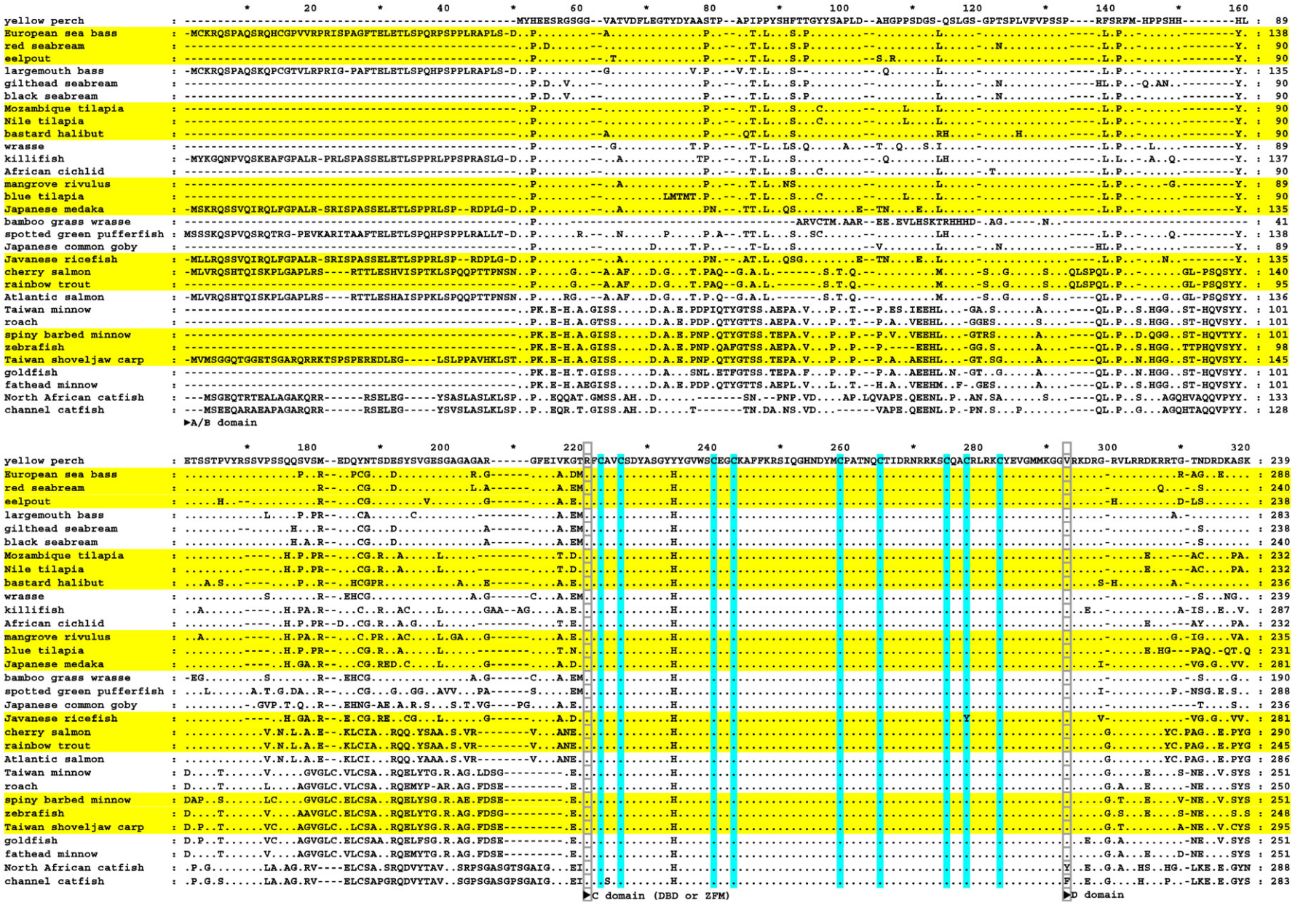
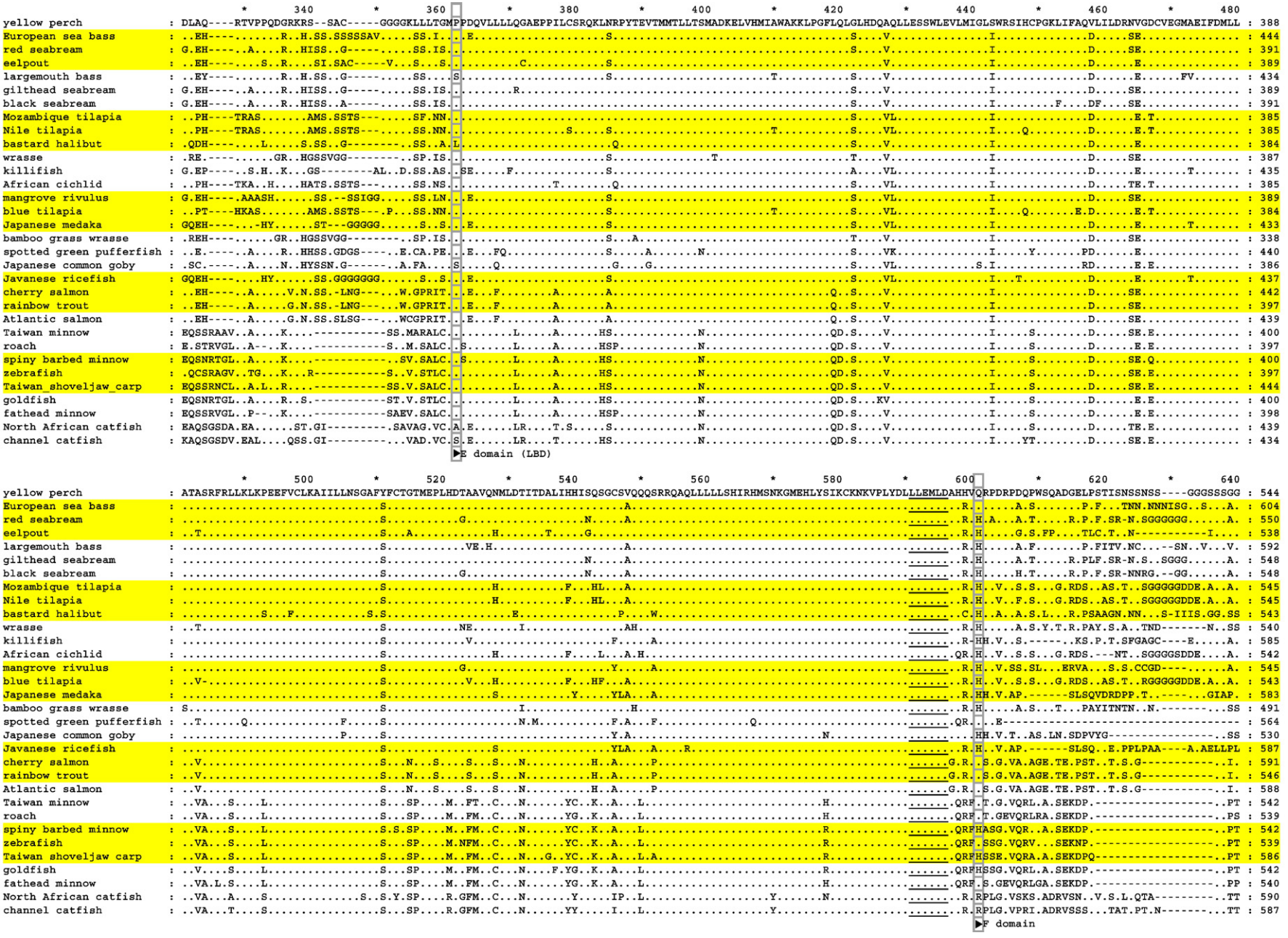


Fig. 1. Nucleotide and deduced amino acid sequences of yellow perch Nucleotide and deduced amino acid sequences of yellow perch *esr1*. The nucleotides (lower row) are numbered on the left and the amino acids (upper row) are numbered on the right. The five functionally independent domain (A/B, C, D, E and F) putative start points are indicated with black arrows above the amino acids. Domain C is referred to as the DNA binding domain (DBD) or the zinc finger motif (ZFM) and domain E is referred to as the ligand binding domain (LBD). The nine conserved cysteines of the C domain are shaded and the ten amino acid residues corresponding to the P- and D-boxes are boxed (Schwabe et al., 1990). The nine amino acids of the E (LBD) domain recognized to be involved in E_2 binding are in bold and the eight amino acid region corresponding to TAF-2 is underlined (Danielian et al., 1992). Two putative polyadenylation signals are in bold and double underlined. Yellow perch *esr1* cDNA sequence is available from the EMBL/GenBank database with Accession DQ984124.


yellow perch
European sea bass
red seabream
eelpout
largemouth bass
gilthead seabream
black seabream
Mozambique tilapia
Nile tilapia
bastard halibut
wrasse
killifish
African cichlid
mangrove rivulus
blue tilapia
Japanese medaka
bamboo grass wrasse
spotted green pufferfish
Japanese common goby
Javanese ricefish
cherry salmon
rainbow trout
Atlantic salmon
Taiwan minnow
roach
spiny barbed minnow
zebrafish
Taiwan shoveljaw carp
goldfish
fathead minnow
North African catfish
channel catfish
▶A/B domain
▶C domain (DBD or ZFM)
▶D domain


E domain (LBD)
F domain

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                                          *       660         *       680         *       700
yellow perch                        : SNSGPRVSRESP-----------------------SRAPTVLQYAESRSDCTRIL---------- : 576
European sea bass          (86.8%)  : .S......H...S---------------------RGPTC.G....GG......H.---------- : 639
red seabream               (86.4%)  : .T......H..-------------------------PTS.G....GG...E..H.---------- : 581
eelpout                    (86.1%)  : .G......HD..-------------------------........GG.I....H.---------- : 570
largemouth bass            (85.8%)  : .S......H...S---------------------RGPTG.G....GG......H.---------- : 627
gilthead seabream          (84.4%)  : .T...Q.NL..-------------------------PTG.G...LR----VHPHP.KPTE------ : 579
black seabream             (83.8%)  : .T..T...L.N-------------------------PTG.G....GR.APSAPHP.KPTE------ : 583
Mozambique tilapia         (82.6%)  : .S...QG.H...RRE----------------NLSRAPTG.G....RG.H......P---------- : 585
Nile tilapia               (82.0%)  : .S...QG.H...RRE----------------NLSRAPTG.G....RG.H......P---------- : 585
bastard halibut            (81.9%)  : AS..H.G.Q...S---------------------RA.TG.....HGG..P...H.---------- : 578
wrasse                     (81.5%)  : P-A.S.A.Q...N---------------------RPPTGH.....GG......H.---------- : 574
killifish                  (81.5%)  : .S....G.GDNLM---------------------RIPS..G....GG.....AQ..---------- : 620
African cichlid            (81.2%)  : .S...QGNH...RCE----------------NLSRAPTG.G....RG.H....P..---------- : 582
mangrove rivulus           (80.9%)  : CS....GIS.N.S---------------------RTPSG......GG..P...NA.---------- : 580
blue tilapia               (79.5%)  : .S...QG.H...RRE----------------NLSRAP.G.G....RG.H......P---------- : 583
Japanese medaka            (79.4%)  : .I.AS.GRI...S---------------------RGPF.......GG..P...PA.QD-------- : 620
bamboo grass wrasse        (79.2%)  : .SA.....L...N---------------------KTPIGQG....GG..P...H.---------- : 526
spotted green pufferfish   (78.5%)  : ------------------------------------------------------------------ :  -
Japanese common goby       (77.6%)  : LS.D..G.STGGG---------------------KMSS......GG.PG...H.A---------- : 564
Javanese ricefish          (76.6%)  : FEAES..RV.A.PPPASFSMEGRVLTAPRPFKTERTVQG.VF.F.GG..VQVGTS---------- : 642
cherry salmon              (71.3%)  : PMR.SQD.HIR-------------------------.PG--....GSPS..QMP.P---------- : 620
rainbow trout              (71.2%)  : PMR.SQD.HIR-------------------------.PGSG....GSPS..QMP.P---------- : 577
Atlantic salmon            (71.0%)  : PMR.SQD.HIR-----------------------.PGSG....GSPS..QMP.PLEQK------ : 623
Taiwan minnow              (65.4%)  : .S.SSS---P.-------------------------.G.GA..PNTAC----PDP---------- : 565
roach                      (65.3%)  : .---------------------------------.G.GA..PNTACLSQ.PEP---------- : 559
spiny barbed minnow        (64.7%)  : .S.SS----L.-----------------------.G.GA..PKTAC.GQ.PDPRPLSYVQSDN : 578
zebrafish                  (64.5%)  : .S.SSSNNSPR-----------------------GGAAA..SNGACHSH.PDP---------- : 569
Taiwan shoveljaw carp      (64.0%)  : .S.SS----P.-----------------------.G.GA.LPNIACH.Q.PDP---------- : 612
goldfish                   (63.8%)  : .S.---------------------------------.G.GT.LPNIACHEQ.PDP---------- : 564
fathead minnow             (63.5%)  : .---------------------------------.G.GAT.PNTGCLSQ.PDP---------- : 560
North African catfish      (60.3%)  : .T.TNQQ.SAP-------------------------PC.AD.PSNPPC..Q.PSP---------- : 620
channel catfish            (60.0%)  : .T.THHP.NG.-------------------------TC.AD.PSNPPGPGQ.PSP---------- : 617
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Fig. 2. Alignment of yellow perch *esr1* deduced amino acid sequence with other teleost *esr1*s. Conserved amino acid residues are indicated with (.), inserted gaps are indicated with (–) and structural domains are identified at the bottom of the alignment with arrows. The nine conserved cysteines of the C domain are shaded (Schwabe et al., 1990) and the eight amino acids corresponding to TAF-2 are underlined (Danielian et al., 1992). The percent (%) listed for each sequence represents percent identity with the yellow perch *esr1* sequence. Sequences were downloaded from the EMBL/GenBank database with the following Accession numbers: European sea bass (*Dicentrarchus labrax*) CAD43599; red seabream (*Pagrus major*) BAA22517; eelpout (*Zoarces viviparus*) AAO66473; largemouth bass (*Micropterus salmoides*) AAG44622; gilthead seabream (*Sparus aurata*) CAB51479; black seabream (*Acanthopagrus schlegelii*) AAL82743; Mozambique tilapia (*Oreochromis mossambicus*) CAK95869; Nile tilapia (*Oreochromis niloticus*) AAD00245; bastard halibut (*Paralichthys olivaceus*) BAB85622; wrasse (*Halichoeres tenuispinis*) AAP72178; killifish (*Fundulus heteroclitus*) BAC76957; African cichlid (*Astatotilapia burtoni*) AAR82891; mangrove rivulus (*Kryptolebias marmoratus*) BAF03498; blue tilapia (*Oreochromis aureus*) CAA63774; Japanese medaka (*Oryzias latipes*) BAA86925; bamboo grass wrasse (*Pseudolabrus japonicus*) ABB96483; spotted green pufferfish (*Tetraodon nigroviridis*) CAG03596; Japanese common goby (*Acanthogobius flavimanus*) BAF46102; Javanese ricefish (*Oryzias javanicus*) AAX13999; cherry salmon (*Oncorhynchus masou*) AAS92970; rainbow trout (*Oncorhynchus mykiss*) CAB45140; Atlantic salmon (*Salmo salar*) AAY25396; Taiwan minnow (*Candidia barbatus*) CAD32175; roach (*Rutilus rutilus*) BAD91035; spiny barbed minnow (*Spinibarbus denticulatus*) ABF56051; zebrafish (*Danio rerio*) BAB16893; Taiwan shoveljaw carp (*Varicorhinus barbatula*) CAD67996; goldfish (*Carassius auratus*) AAL12298; fathead minnow (*Pimephales promelas*) AAU87498; North African catfish (*Clarias gariepinus*) CAC37560; and channel catfish (*Ictalurus punctatus*) AAG24543.

African clawed frog, silurana tropicalis and zebra finch; 50.2% Japanese quail; 50.0% chicken; 49.7% pig; 49.2% cattle; 49.1% golden hamster; 49.0% rat; 48.9% leopard gecko; 48.6% human; 48.5% horse; and 47.9% praire vole. A phylogenetic tree of *esr1* proteins in teleosts, using the African clawed frog (*Xenopus laevis*) as an outgroup, was constructed from alignment results (Fig. 3). This tree indicates an early split of Neoteleostei from other Teleostei (Cypriniformes and Siluriformes) with the exception of Salmoniformes (salmon and trout) which splits from Neoteleostei separately. The moderate bootstrap value for the split between Cichlidae and Cyprinodontidae/Belonidae (666) is probably derived of the differences in the length of the A/B domain between many of these *esr1* protein sequences. The low bootstrap values for sister groupings within Perciformes (546, 518) may result from highly similar protein sequences.

3.2. Esr2a

Yellow perch *esr2a* cDNA (Accession DQ984125) was found to consist of 2462 bp containing an ORF of 1668 bp, a 546 bp 5′-UTR and a 248 bp 3′-UTR. The designated open reading frame encoded a protein of 555 amino acids consisting of 5 domains common to estrogen receptors (Fig. 4). The isoelectric point and molecular mass of the *esr2a* peptide, as calculated by Vector NTI BioPlot, are 8.47 and 61,864.83 Da, respectively. Comparison of the deduced amino acid sequence of the yellow perch *esr2a* with other known teleost ERβ as is shown in Fig. 5, with percent identity (listed in the last row of the figure) determined using DNASTAR Lasergene v. 7.2.0. Yellow perch *esr2a* was also determined to have percent identities with the following vertebrate ERβs: 62.0% European sea bass ERβ2; 60.3% largemouth bass ERβ; 59.6% Atlantic croaker ERβ; 59.5% gilthead seabream ERβ2; 58.5% wrasse ERβ[b]; 58.4% Nile tilapia ERβ2; 58.2% channel catfish ERβ[b]; 57.8% killifish ERβB and Japanese quail; 57.6% chicken; 57.4% mangrove rivulus ERβ[b]; 56.8% sheep; 56.5% leopard gecko; 56.4% zebrafish ERβ1; 56.0% cattle; 55.2% Taiwan shoveljaw carp ERβ1; 55.1% goldfish ERβ2 and pig; 54.9% human; 54.8% fathead minnow ERβ[b]; 54.7% white-tufted-ear marmoset; 54.5% Taiwan minnow ER[βb] and roach ERβ[b]; 54.4% spiny dogfish; 53.9% mouse and rat; 53.5% silurana tropicalis; and 52.9% common starling. A phylogenetic tree of teleost *esr2* proteins, with the spiny dogfish shark (*Squalus acanthias*) as an outgroup, was constructed from alignment results (Fig. 6). The tree indicates a distinct split between the two types of fish *esr2* proteins very early in the teleost lineage, with the bottom group termed *esr2a* and the top group termed *esr2b*. The *esr2a* group shows the order Anguilliformes (eels) splitting first followed by Cypriniformes (goldfish, carp etc.). Strangely, rainbow trout (*Oncorhynchus mykiss*: Salmoniformes) is grouped with the Cypriniformes, while Atlantic salmon (*Salmo salar*: Salmoniformes) splits before the divergence of Neoteleostei. There is low branch support for the Pleuronectiformes split (384), likely reflecting the lack of *esr2a* sequences for this group. The *esr2b* group shows a clear and early split between Neoteleostei and the other teleosts and there is high branch support for such groupings. Within Neoteleostei, Nile tilapia (*Oreochromis niloticus*: Cichlidae) splits with the Cyprinodontiformes from the Perciformes, which is an odd grouping, but probably reflects the absence of additional sequence information for these groups.

3.3. Cyp19a1a

Yellow perch *cyp19a1a* cDNA (Accession DQ984126) was found to consist of 1859 bp containing an ORF of 1557 bp, a 68 bp 5′-UTR and a 234 bp 3′-UTR. The designated open reading frame encoded a protein of 518 amino acids containing four conserved regions present in all steroidogenic cytochrome *P-450*s (Fig. 7). The isoelectric point and molecular mass of the *cyp19a1a* peptide, as calculated by Vector NTI BioPlot, are 8.51 and 58,535.77 Da, respectively.

Comparison of the deduced amino acid sequence of the yellow perch *cyp19a1a* with other known teleost *cyp19a1a*s is shown in Fig. 8, with percent identity (listed in the last row of the figure) determined using DNASTAR Lasergene v. 7.2.0. Yellow perch *cyp19a1a* was also determined to have percent identities with the following vertebrate ovarian aromatases: 55.2% Japanese firebelly newt; 54.4% Iberian ribbed newt; 54.0% wrinkled frog; 53.8% chicken; 53.3% Atlantic stingray, African clawed frog and silurana tropicalis; 52.9% zebra finch; 52.5% pig; 52.2% white-tufted-ear marmoset and rabbit; 51.8% sheep; 51.1% American alligator and red-eared slider turtle; and 51.0% horse. A phylogenetic tree of teleost *cyp19a1a* proteins, with Atlantic stingray (*Dasyatis sabina*) as an outgroup, was constructed from alignment results (Fig. 9). The tree shows an early split of the Neoteleostei and the Salmoniformes within the teleosts followed by a "peeling off" of other Neoteleosti groups. Immediately after the Salmoniformes split, the broad barred goby (*Gobiodon histrio*: Perciformes) splits followed by tongue sole (*Cynoglossus semilaevis*: Pleuronectiformes) and then spotted green pufferfish (*Tetraodon nigroviridis*: Tetraodontiformes). The moderate to high bootstrap values (797, 959) associated with these splits and some unique substitutions in these sequences, as shown in Fig. 8, support their early and singular splits from Neoteleostei. Several sister groups then split from the main tree, with unusually low bootstrap values, and ultimately yellow perch is grouped near the groupers and Atlantic croaker (*Micropogonias undulates*). While it is difficult to interpret all the node/split values, other studies have reported low bootstrap values for *cyp19a1a* proteins (Nunez and Applebaum, 2006).

3.4. Tissue expression

The primers in Table 2 were used to generate full length coding region PCR products for *esr1*, *esr2a* and *cyp19a1a* of 1781, 1718 and 1605 bp in length, respectively, and whose identities were confirmed by DNA sequencing. These primers were then used to determine sex-specific tissue expression (Fig. 10). Both *esr1* and *esr2a* showed the highest expression in female tissues, particularly liver and ovary. Female liver and

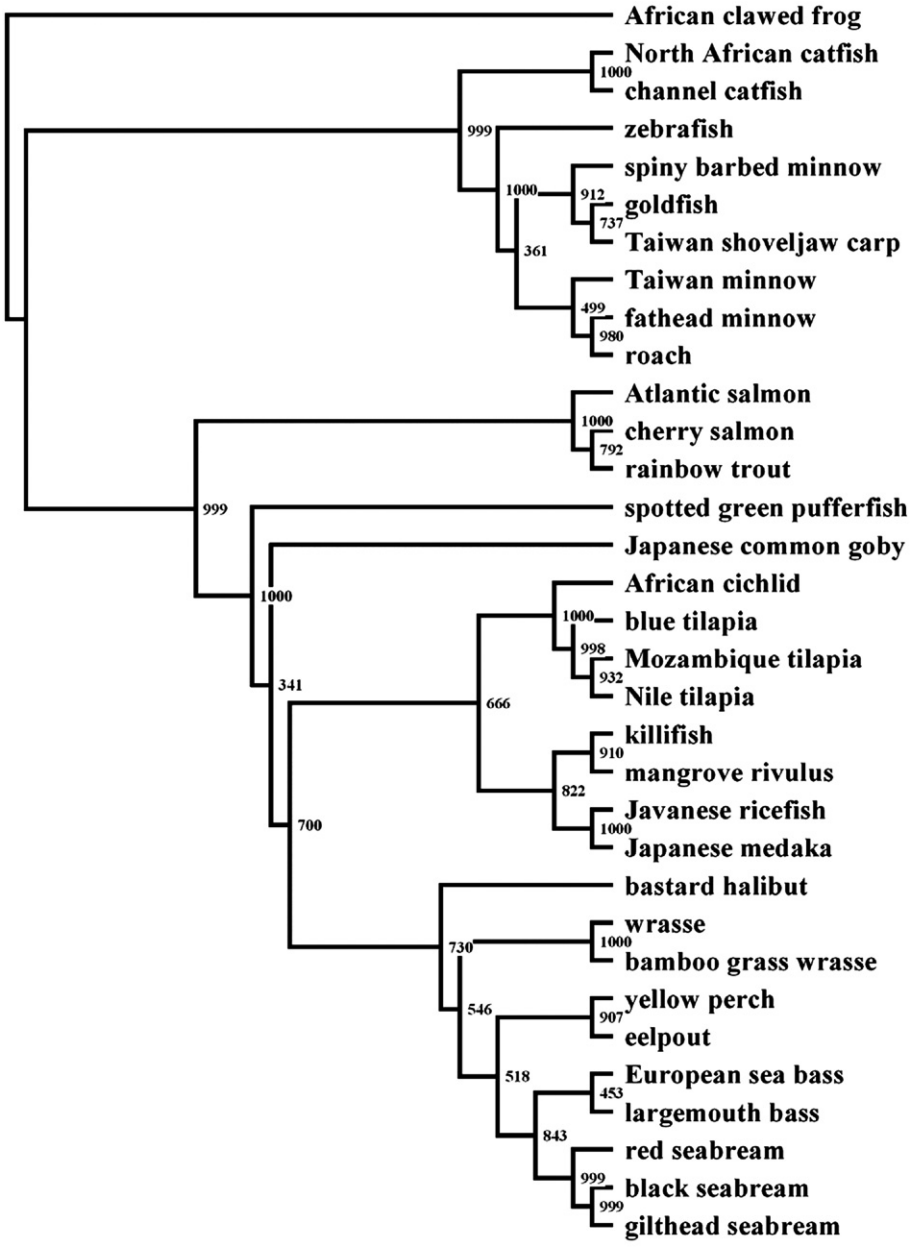


Fig. 3. Phylogenetic tree of *esr1* amino acid sequences. Tree produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with African clawed frog (*Xenopus laevis*) ERα (Accession AAQ84782) as an outgroup representing the closest available species not in the group Teleostei. See Fig. 2 for EMBI/GenBank Accession numbers.

ovary tissues showed substantial expression of *esr1* and spleen tissue was the only other female tissue to show *esr1* expression. Female liver and ovary tissues also showed substantial expression of *esr2a* with low expression in stomach and kidney tissues and oocytes. Male spleen, liver and stomach tissues showed moderate to low expression of *esr1* and male kidney, liver, and spleen tissues showed moderate expression of *esr2a*. *Esr2a* expression was low in male stomach, heart, gill and brain tissues. *Cyp19a1a* showed a very global expression pattern in both male and female yellow perch tissues. Female spleen and liver along with oocytes showed high expression of *cyp19a1a*, whereas female brain, gill, stomach and ovary tissues showed moderate to low expression of *cyp19a1a*. Male pituitary, gill and spleen tissues showed moderate to high expression of *cyp19a1a* while male heart, stomach and kidney tissues showed low expression.

4. Discussion

In this study, the full length cDNA sequences for yellow perch *esr1*, *esr2a* and *cyp19a1a* have been characterized, phylogenetic analyses presented on the deduced amino acid sequences and sex-specific tissue expression patterns determined. This study has shown a clear sex-specific expression pattern for *esr1*, *esr2a* and *cyp19a1a* in several yellow perch tissues, particularly the liver which is a key tissue in the growth

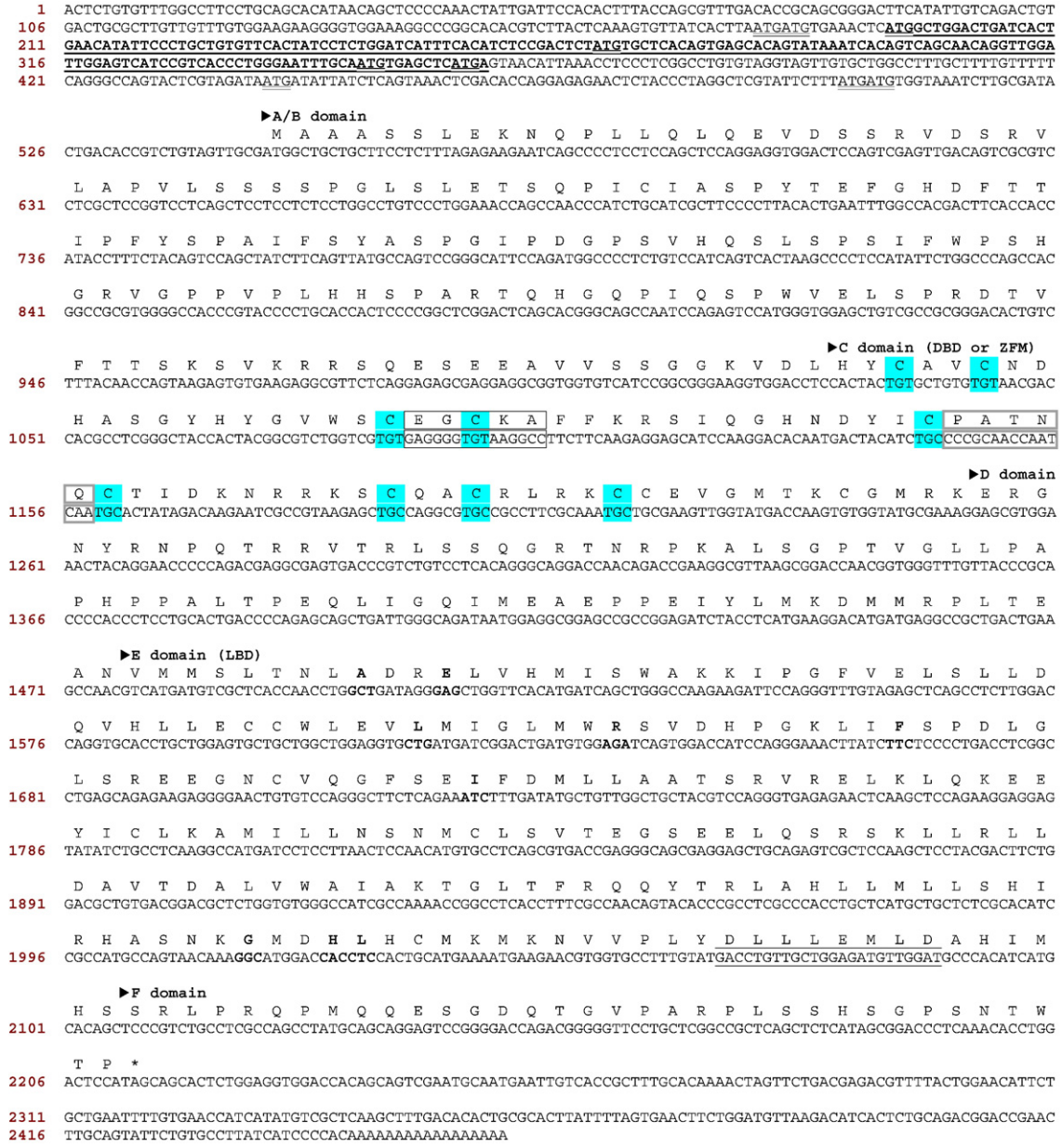
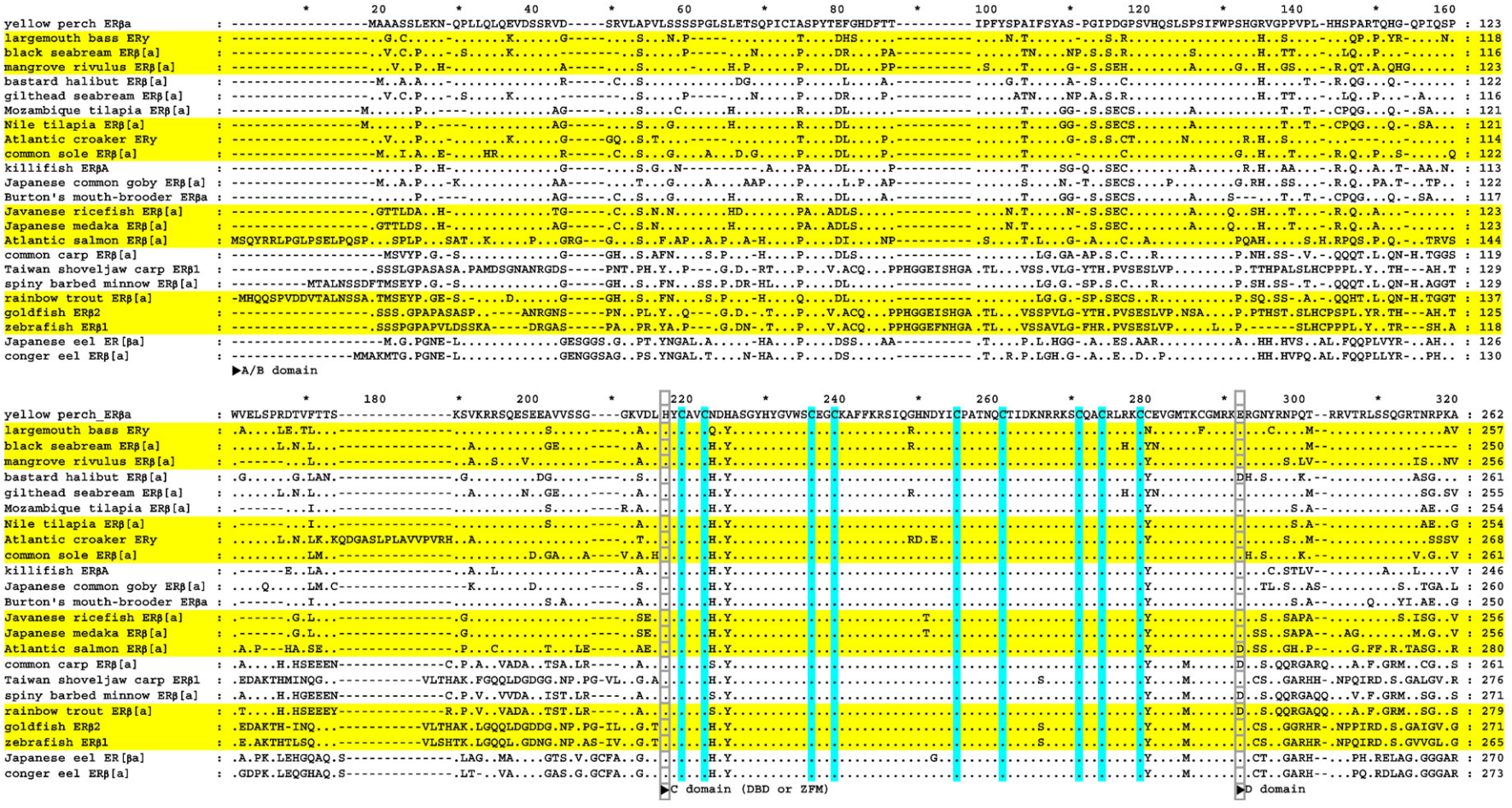
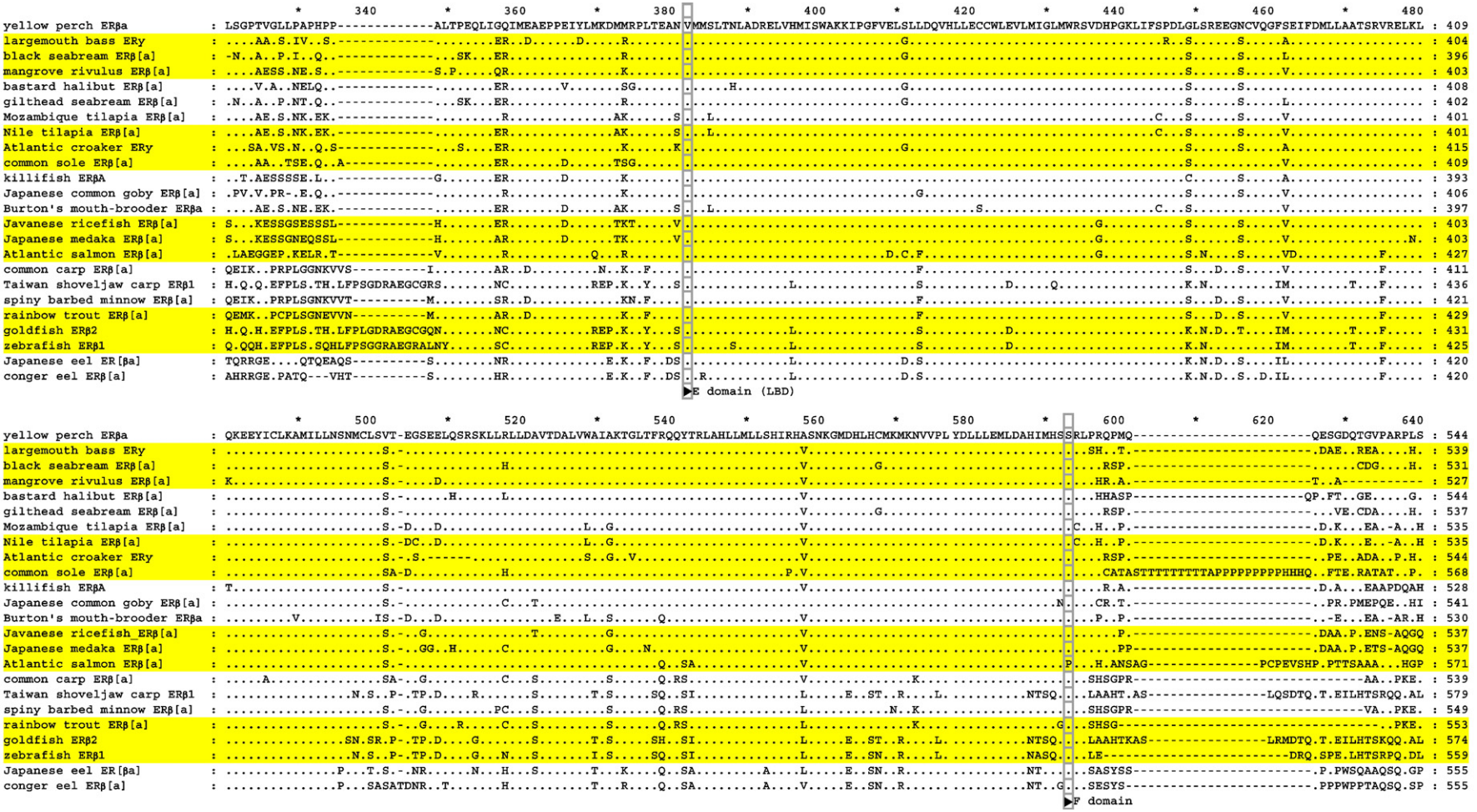


Fig. 4. Nucleotide and deduced amino acid sequences of yellow perch *esr2a* cDNA. The nucleotides (lower row) are numbered on the left and the amino acids (upper row) are numbered on the right. The five functionally independent domain (A/B, C, D, E and F) putative start points are indicated with black arrows above the amino acids. Domain C is also referred to as the DNA binding domain (DBD) or the zinc finger motif (ZFM) and domain E is also referred to as the ligand binding domain (LBD). The nine conserved cysteines of the C domain are shaded and the ten amino acid residues corresponding to the P- and D-boxes are boxed (Schwabe et al., 1990). The nine amino acids of the E (LBD) domain recognized to be involved in E_2 binding are in bold and the eight amino acid region corresponding to TAF-2 is underlined (Danielian et al., 1992). The nine supplemental ATG initiation codons in the 5′-UTR are double underlined and the single in-frame ORF beginning at base 193 is underlined in bold. Yellow perch *esr2a* cDNA sequence is available from the EMBL/GenBank database with Accession DQ984125.

axis. These sequences, and the ones presented in Lynn and Shepherd (2007), are essential to future studies aimed at determining the mechanisms of estrogen-stimulated SDD in yellow perch.

Esr1 cDNA of the yellow perch was cloned by directional RACE procedures and was shown to encode a protein of 576 amino acids. The yellow perch *esr1* retained the conserved cysteines in the C domain responsible for the formation of the zinc fingers, the P and D box regions associated with DNA binding and the nine residues and transcriptional activation factor-2 (TAF-2) associated with E_2 binding (Danielian et al., 1992; Ekena et al., 1996; Brzozowski et al., 1997). Unlike European sea bass (Halm et al., 2004) and goldfish (Choi and Habibi, 2003) ERαs, yellow perch *esr1* does not appear to contain the TAF-1. There are several amino acid substitutions that are unique to the yellow perch, such as the Tyr_{155} in the C domain where all other known fish have a His residue, but several other species show similar unique substitutions. The most interesting unique substitution is the wrasse (*Halichoeres tenuispinis*) Thr_{308} substitution (Kim et al., 2002) for yellow perch Ala_{309} which is reported to be one of the key residues in the E domain responsible for E_2 binding (Brzozowski et al.,


yellow perch ERβa
largemouth bass ERy
black seabream ERβ[a]
mangrove rivulus ERβ[a]
bastard halibut ERβ[a]
gilthead seabream ERβ[a]
Mozambique tilapia ERβ[a]
Nile tilapia ERβ[a]
Atlantic croaker ERy
common sole ERβ[a]
killifish ERβA
Japanese common goby ERβ[a]
Burton's mouth-brooder ERβa
Javanese ricefish ERβ[a]
Japanese medaka ERβ[a]
Atlantic salmon ERβ[a]
common carp ERβ[a]
Taiwan shoveljaw carp ERβ1
spiny barbed minnow ERβ[a]
rainbow trout ERβ[a]
goldfish ERβ2
zebrafish ERβ1
Japanese eel ER[βa]
conger eel ERβ[a]
▶A/B domain
▶C domain (DBD or ZFM)
▶D domain


yellow perch ERβa
largemouth bass ERy
black seabream ERβ[a]
mangrove rivulus ERβ[a]
bastard halibut ERβ[a]
gilthead seabream ERβ[a]
Mozambique tilapia ERβ[a]
Nile tilapia ERβ[a]
Atlantic croaker ERy
common sole ERβ[a]
killifish ERβA
Japanese common goby ERβ[a]
Burton's mouth-brooder ERβa
Javanese ricefish ERβ[a]
Japanese medaka ERβ[a]
Atlantic salmon ERβ[a]
common carp ERβ[a]
Taiwan shoveljaw carp ERβ1
spiny barbed minnow ERβ[a]
rainbow trout ERβ[a]
goldfish ERβ2
zebrafish ERβ1
Japanese eel ER[βa]
conger eel ERβ[a]
E domain (LBD)
yellow perch ERβa
largemouth bass ERy
black seabream ERβ[a]
mangrove rivulus ERβ[a]
bastard halibut ERβ[a]
gilthead seabream ERβ[a]
Mozambique tilapia ERβ[a]
Nile tilapia ERβ[a]
Atlantic croaker ERy
common sole ERβ[a]
killifish ERβA
Japanese common goby ERβ[a]
Burton's mouth-brooder ERβa
Javanese ricefish_ERβ[a]
Japanese medaka ERβ[a]
Atlantic salmon ERβ[a]
common carp ERβ[a]
Taiwan shoveljaw carp ERβ1
spiny barbed minnow ERβ[a]
rainbow trout ERβ[a]
goldfish ERβ2
zebrafish ERβ1
Japanese eel ER[βa]
conger eel ERβ[a]
F domain

```
                                                  *       660         *       680
yellow perch ERβa                        : SH-SGPSNTWTP---------------------------- : 555
largemouth bass ERγ          (84.0%) : .G-.C.......----GGGEPQ------------------ : 556
black seabream ERβ[a]        (83.4%) : PGP.........SSTGGRGEPQ------------------ : 553
mangrove rivulus ERβ[a]      (83.2%) : --AA.........SSVGVGGEAQYSE---------------- : 550
bastard halibut ERβ[a]       (83.0%) : .G-N.S......STSGDGGEPQ------------------ : 565
gilthead seabream ERβ[a]     (82.3%) : PGT.........SCTGGRGEPQ------------------ : 559
Mozambique tilapia ERβ[a]    (82.2%) : .SAG........SSARAGGESQ------------------ : 557
Nile tilapia ERβ[a]          (82.0%) : .SAG........SSARAGGESQ------------------ : 557
Atlantic croaker ERγ         (81.7%) : .G-....Y....SSSEGAGEPQ------------------ : 565
common sole ERβ[a]           (81.6%) : CG-KSSP.....GSNRDAGEAQ------------------ : 589
killifish ERβA               (79.8%) : CSAA........SGAGVAGEAQCSD---------------- : 553
Japanese common goby ERβ[a]  (79.7%) : .P-......C..SEDENQPSETIKTPQ-------------- : 567
Burton's mouth-brooder ERβa  (79.5%) : .SA.........SSARAGGEPQ------------------ : 552
Javanese ricefish ERβ[a]     (78.4%) : RSCW-V.KA...SSPGAAGAAQKSDQN-------------- : 563
Japanese medaka ERβ[a]       (77.4%) : RSCCDV.KA..TSSAGTAEEPQKSD---------------- : 562
Atlantic salmon ERβ[a]       (71.0%) : PAAEASL..R.HWTAGTPVERQW------------------ : 594
common carp ERβ[a]           (68.3%) : KGVQEALTRT.QSGGTLAGP--------------------- : 559
Taiwan shoveljaw carp ERβ1   (68.3%) : KESYQEPWHSPQAEETVDKILH---CSLHRVDMDTD----- : 612
spiny barbed minnow ERβ[a]   (67.2%) : KAIQEAFACT.QHG-----P--------------------- : 564
rainbow trout ERβ[a]         (67.2%) : .GVQEA.L.VLKNDL------------------------- : 568
goldfish ERβ2                (67.2%) : KESNQD.RHSPQAEGTVDKTLHRVDKTLHRVDVDTD----- : 610
zebrafish ERβ1               (66.4%) : KDSDQE.PHSPRAEEMVNKTLH---SSLLREDMDTN----- : 592
Japanese eel ER[βa]          (61.5%) : PPSCSGECPCP.KESSTI----------------------- : 573
conger eel ERβ[a]            (60.9%) : QPSCSGEGPCP.KESITAAVFGHGEDRVIPGLHTGTTSRRD : 596
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Fig. 5. Alignment of yellow perch *esr2a* deduced amino acid sequence with other teleost *esr2a*s. Conserved amino acid residues are indicated with (.), inserted gaps are indicated with(–) and structural domains are identified at the bottom of the alignment with arrows. The nine conserved cysteines of the C domain are shaded (Schwabe et al., 1990) and the eight amino acids corresponding to TAF-2 are underlined (Danielian et al., 1992). The percent (%) listed for each sequence represents percent identity with the yellow perch *esr2a* sequence. Sequences were downloaded from the EMBL/GenBank database with the following Accession numbers: largemouth bass ERγ (*Micropterus salmoides*) AAO39211; black seabream ERβ[a] (*Acanthopagrus schlegelii*) AAL82742; mangrove rivulus ERβ[a] (*Kryptolebias marmoratus*) BAF03497; bastard halibut ERβ[a] (*Paralichthys olivaceus*) BAB85623; gilthead seabream ERβ[a] (*Sparus aurata*) AAD31033; Mozambique tilapia ERβ[a] (*Oreochromis mossambicus*) CAK95870; Nile tilapia ERβ[a] (*Oreochromis niloticus*) AAD00246; Atlantic croaker ERγ (*Micropogonias undulates*) AAG16712; common sole ERβ[a] (*Solea solea*) CAL09961; killifish ERβA (*Fundulus heteroclitus*) AAU44352; Japanese common goby ERβ[a] (*Acanthogobius flavimanus*) BAF46103; Burton's mouth-brooder ERβa (*Astatotilapia burtoni*) ABI18966; Javanese ricefish ERβ[a] (*Oryzias javanicus*) AAX14000; Japanese medaka ERβ[a] (*Oryzias latipes*) BAB79705; Atlantic salmon ERβ[a] (*Salmo salar*) AAR92486; common carp ERβ[a] (*Cyprinus carpio*) BAB91218; Taiwan shoveljaw carp ERβ2 (*Varicorhinus barbatula*) CAD67997; spiny barbed minnow ERβ[a] (*Spinibarbus denticulatus*) ABF56052; rainbow trout ERβ[a] (*Oncorhynchus mykiss*) CAC06714; goldfish ERβ1 (*Carassius auratus*) AAD26921; zebrafish ERβ2 (*Danio rerio*) CAC93849; Japanese eel ER[βa] (*Anguilla japonica*) BAA19851; and conger eel ERβ[a] (*Conger myriaster*) BAD02929.

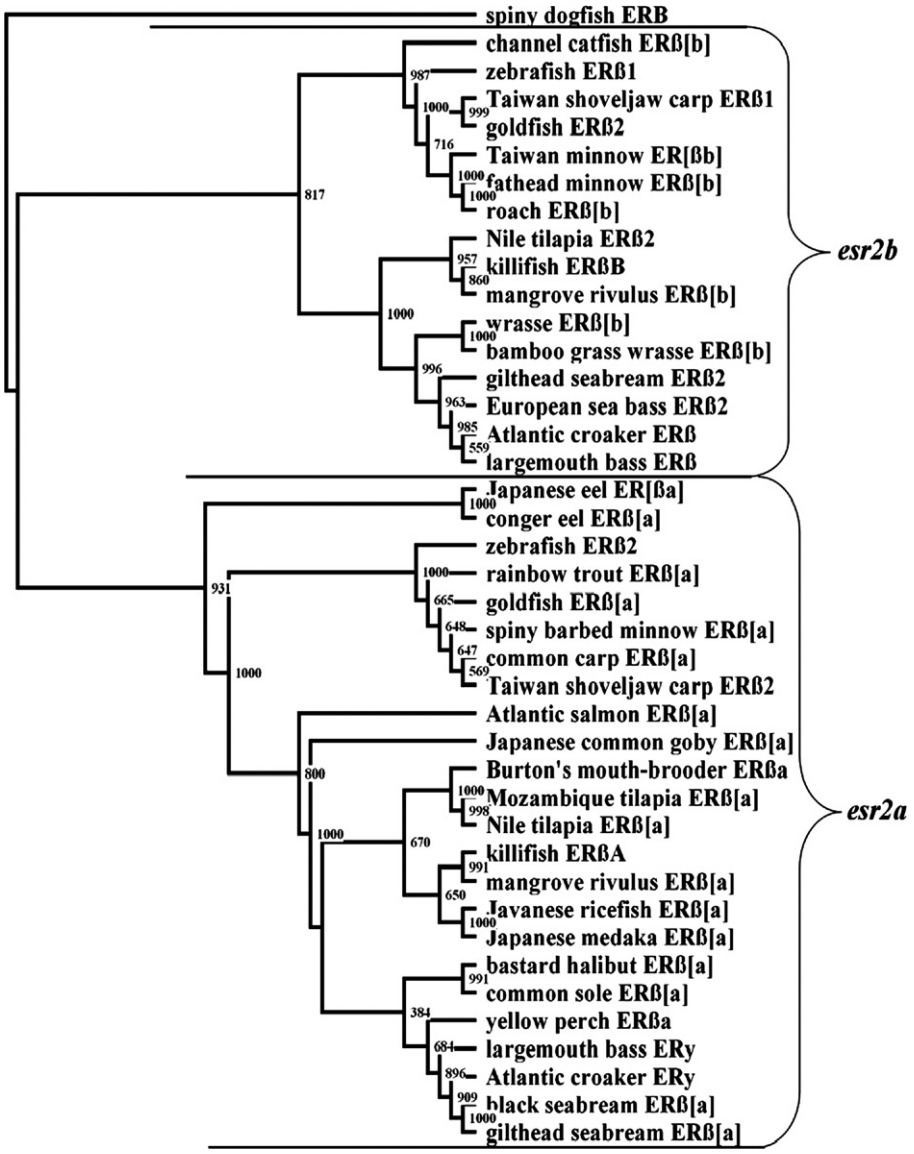


Fig. 6. Phylogenetic tree of *esr2* amino acid sequences. Tree produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with spiny dogfish shark (*Squalus acanthias*) ERβ (Accession AAK57823) as an outgroup representing the closest available species not in the group Teleostei. See Fig. 5 for EMBI/GenBank Accession numbers except: channel catfish ERβ[b] (*Ictalurus punctatus*) AAF63157; zebrafish ERβ1 (*Danio rerio*) CAC93848; Taiwan shoveljaw carp ERβ1 (*Varicorhinus barbatula*) CAC85366; goldfish ERβ2 (*Carassius auratus*) AAF35170; Taiwan minnow ER[βb] (*Candidia barbatus*) CAC85356; fathead minnow ERβ[b] (*Pimephales promelas*) AAT45195; roach ERβ[b] (*Rutilus rutilus*) BAD91036; Nile tilapia ERβ2 (*Oreochromis niloticus*) ABE73151; killifish ERβB (*Fundulus heteroclitus*) AAU44353; mangrove rivulus ERβ[b] (*Kryptolebias marmoratus*) ABC68616; wrasse ERβ[b] (*Halichoeres tenuispinis*) AAP72179; bamboo grass wrasse ERβ[b] (*Pseudolabrus japonicus*) ABB96484; gilthead seabream ERβ2 (*Sparus aurata*) CAE30470; European sea bass ERβ2 (*Dicentrarchus labrax*) CAD33852; Atlantic croaker ERβ (*Micropogonias undulates*) AAG16711; and largemouth bass ERβ (*Micropterus salmoides*) AAO39210.

1997). Ekena et al. (1996) reported that a mutation of human ERα Met_{528}, corresponding to yellow perch *esr1* Ile_{487}, to an Ala residue caused an 11-fold reduction in E_2-induced transcription. However, Matthews et al. (2001) found that individually mutating the human ERα Leu_{349} and Met_{528} residues to the rainbow trout ERα Met_{317} and Ile_{496} residues, respectively, (corresponding to yellow perch *esr1* Met_{308} and Ile_{487}, which are conserved in all teleosts) did not significantly reduce the E_2 binding affinity at four different temperatures. Analyses indicate that yellow perch *esr1* shares the highest percent identity with European sea bass (*D. labrax*), but has the closest phylogenetic relationship to eelpout (*Zoarces viviparous*).

Esr2a cDNA of the yellow perch was cloned by directional RACE procedures and encodes a protein of 555 amino acids. The yellow perch *esr2a* sequence showed that the 546 bp 5′-UTR contained a total of nine supplemental ATG initiation codons and the single in-frame supplemental codon had a short open reading frame (ORF) of 55 amino acids. Small ORFs with uncharacterized functions have also been identified in the 5′-UTR of ER cDNAs from many species of fish (Tchoudakova et al., 1999; Halm et al., 2004; Pinto et al., 2006). In human and mouse ERαs, the small upstream ORFs are involved in the regulation of the levels of translation of the ER protein, providing the potential to regulate expression (Koš et al., 2002).

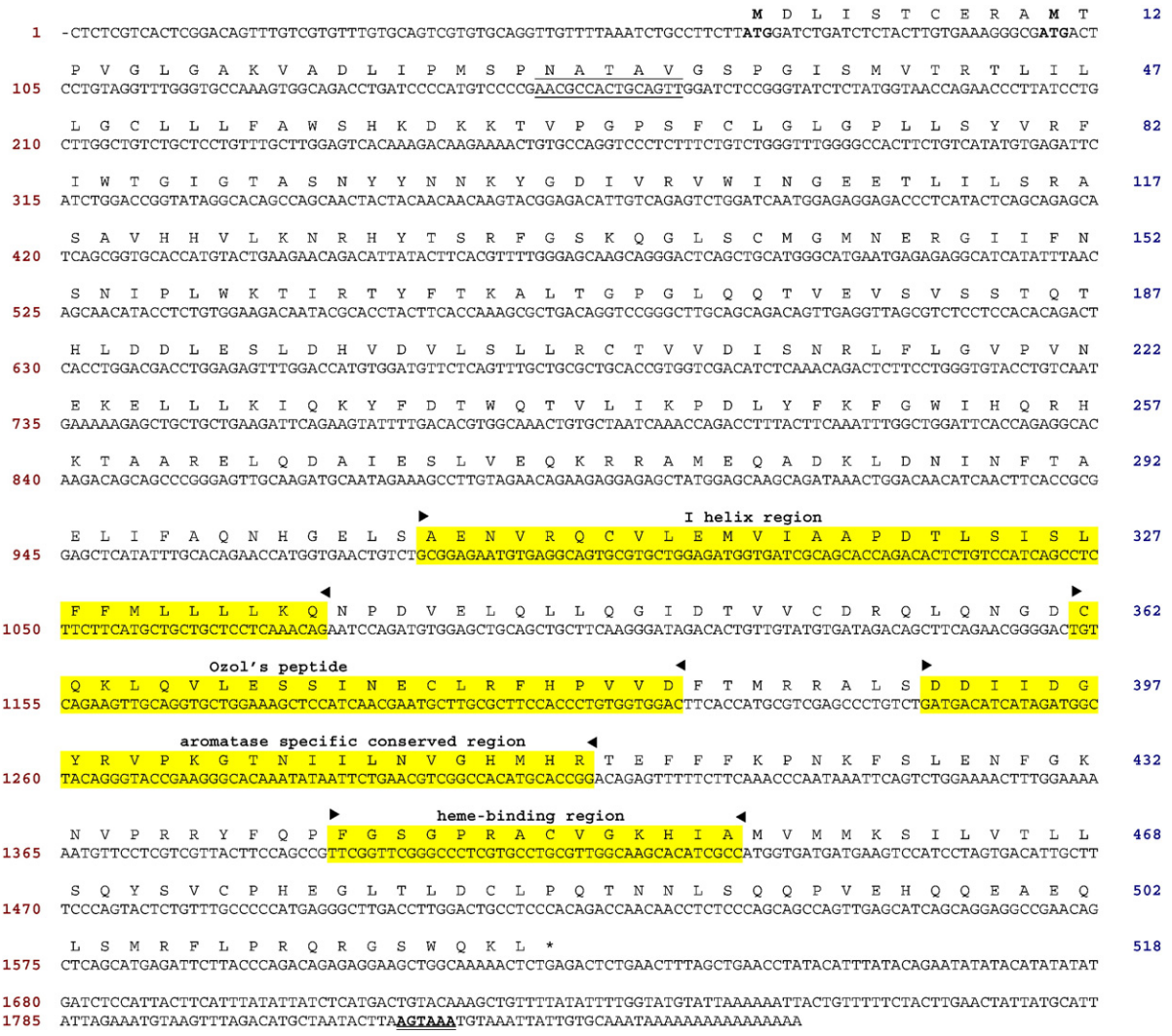
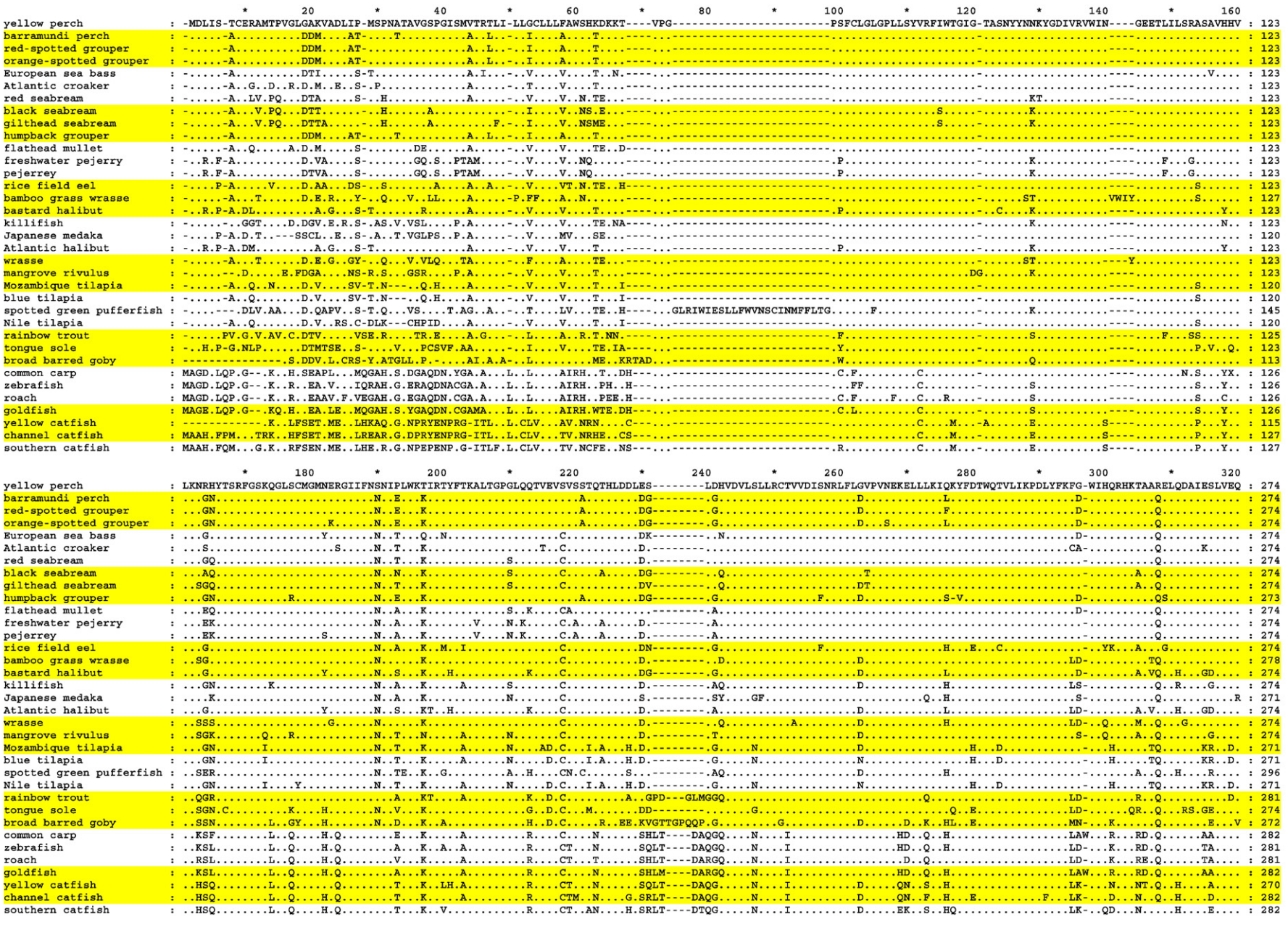


Fig. 7. Nucleotide and deduced amino acid sequences of yellow perch *cyp19a1a* cDNA. The nucleotides (lower row) are numbered on the left and the amino acids (upper row) are numbered on the right. The two in-frame initiation codons are in bold and a potential polyadenylation signal is in bold and double underlined. A putative *N*-glycosylation site is underlined and the following conserved regions of all steroidogenic cytochrome *P-450*s are shaded and labeled in bold with arrows above the amino acid sequence: I helix region, Ozol's peptide, aromatase specific conserved region and the heme-binding region (Graham-Lorence et al., 1991). Yellow perch *cyp19a1a* cDNA sequence is available from the EMBL/GenBank database with Accession DQ984126.

The yellow perch *esr2a* also retained the conserved cysteines in the C domain responsible for the formation of the zinc fingers, the P and D box regions associated with DNA binding and the nine residues and TAF-2 associated with E_2 binding (Ekena et al., 1996; Brzozowski et al., 1997). As with yellow perch *esr1*, yellow perch *esr2a* does not appear to contain the TAF-1.

Fig. 8. Alignment of yellow perch *cyp19a1a* deduced amino acid sequence with other teleost *cyp19a1a*s. Conserved amino acid residues are indicated with (.) and inserted gaps are indicated with (–). Structural domains conserved in all steroidogenic cytochrome *P-450*s are identified at the bottom of the alignment with arrows (IHR-I helix region; OP-Ozol's peptide; ASCR-aromatase specific conserved region; HBR-heme-binding region). The percent (%) listed for each sequence represents percent identity with the yellow perch *cyp19a1a* sequence. Sequences were downloaded from the EMBL/GenBank database with the following Accession numbers: barramundi perch (*Lates calcarifer*) AAV91179; red-spotted grouper (*Epinephelus akaara*) AAS58448; orange-spotted grouper (*Epinephelus coioides*) AR97601; European sea bass (*Dicentrarchus labrax*) CAC21712; Atlantic croaker (*Micropogonias undulatus*) ABA26927; red seabream (*Pagrus major*) BAB82524; black seabream (*Acanthopagrus schlegelii*) AAP23236; gilthead seabream (*Sparus aurata*) AAL27699; humpback grouper (*Cromileptes altivelis*) AAV91178; flathead mullet (*Mugil cephalus*) AAW72732; freshwater pejerrey (*Odontesthes hatcheri*) ABK41198; pejerrey (*Odontesthes bonariensis*) ABK30807; rice field eel (*Monopterus albus*) AAS94314; bamboo grass wrasse (*Pseudolabrus japonicus*) ABB96485; bastard halibut (*Paralichthys olivaceus*) BAA74777; killifish (*Fundulus heteroclitus*) AAR97268; Japanese medaka (*Oryzias latipes*) BAA11657; Atlantic halibut (*Hippoglossus hippoglossus*) CAC36394; wrasse (*Halichoeres tenuispinis*) AAR37048; mangrove rivulus (*Kryptolebias marmoratus*) ABC68614; Mozambique tilapia (*Oreochromis mossambicus*) AAD31031; blue tilapia (*Oreochromis aureus*) ABB89869; spotted green pufferfish (*Tetraodon nigroviridis*) AF99837; Nile tilapia (*Oreochromis niloticus*) AAO62625; rainbow trout (*Oncorhynchus mykiss*) 1806325A; tongue sole (*Cynoglossus semilaevis*) ABL74474; broad barred goby (*Gobiodon histrio*) AAV91177; common carp (*Cyprinus carpio*) ABF82249; zebrafish (*Danio rerio*) AAK00643; roach (*Rutilus rutilus*) BAD91037; goldfish (*Carassius auratus*) AAC14013; yellow catfish (*Pseudobagrus fulvidraco*) AAW65999; channel catfish (*Ictalurus punctatus*) AAB32613; southern catfish (*Silurus meridionalis*) AAP83133.


yellow perch
barramundi perch
red-spotted grouper
orange-spotted grouper
European sea bass
Atlantic croaker
red seabream
black seabream
gilthead seabream
humpback grouper
flathead mullet
freshwater pejerry
pejerrey
rice field eel
bamboo grass wrasse
bastard halibut
killifish
Japanese medaka
Atlantic halibut
wrasse
mangrove rivulus
Mozambique tilapia
blue tilapia
spotted green pufferfish
Nile tilapia
rainbow trout
tongue sole
broad barred goby
common carp
zebrafish
roach
goldfish
yellow catfish
channel catfish
southern catfish

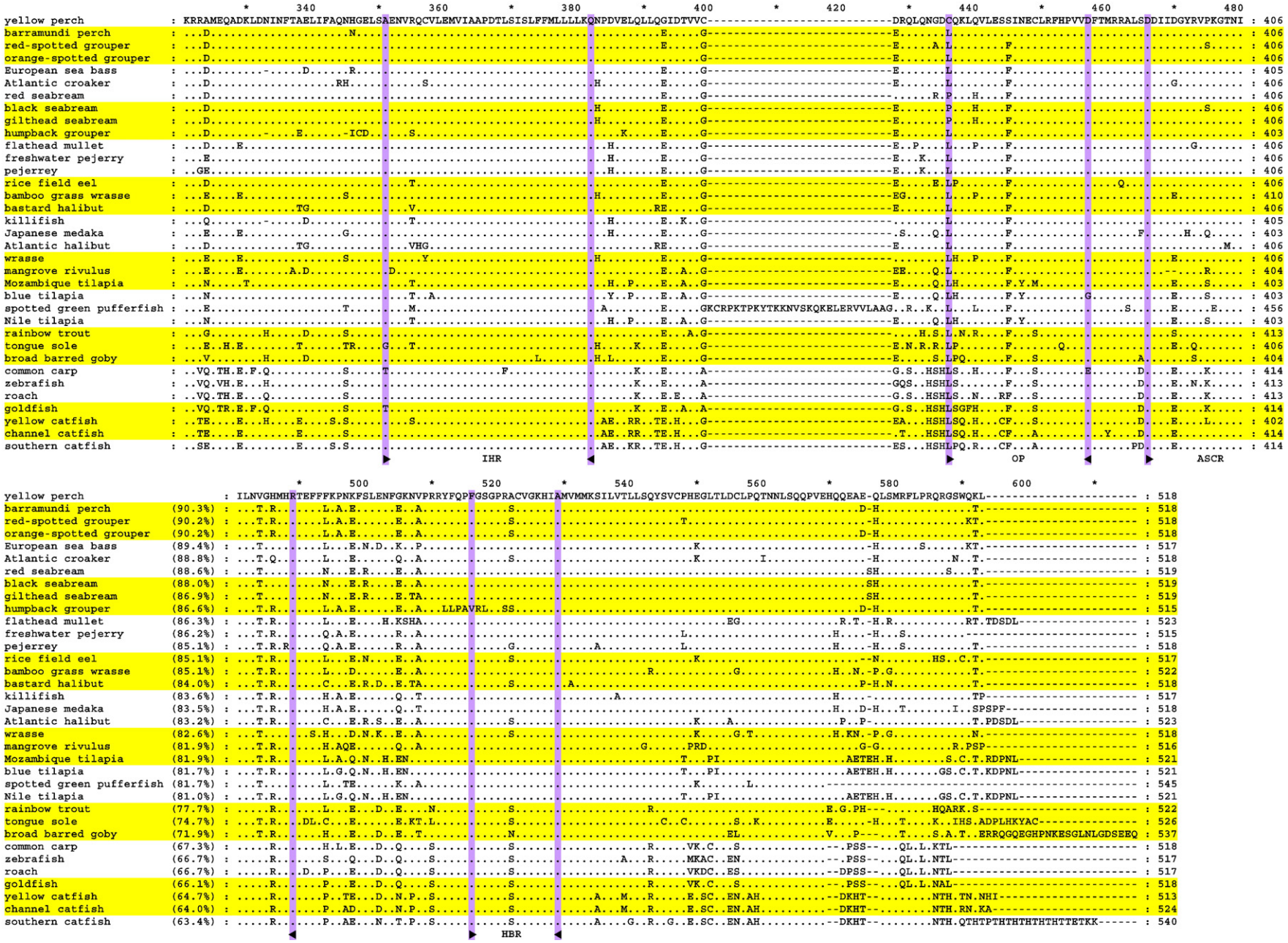


Fig. 8 (*continued*).

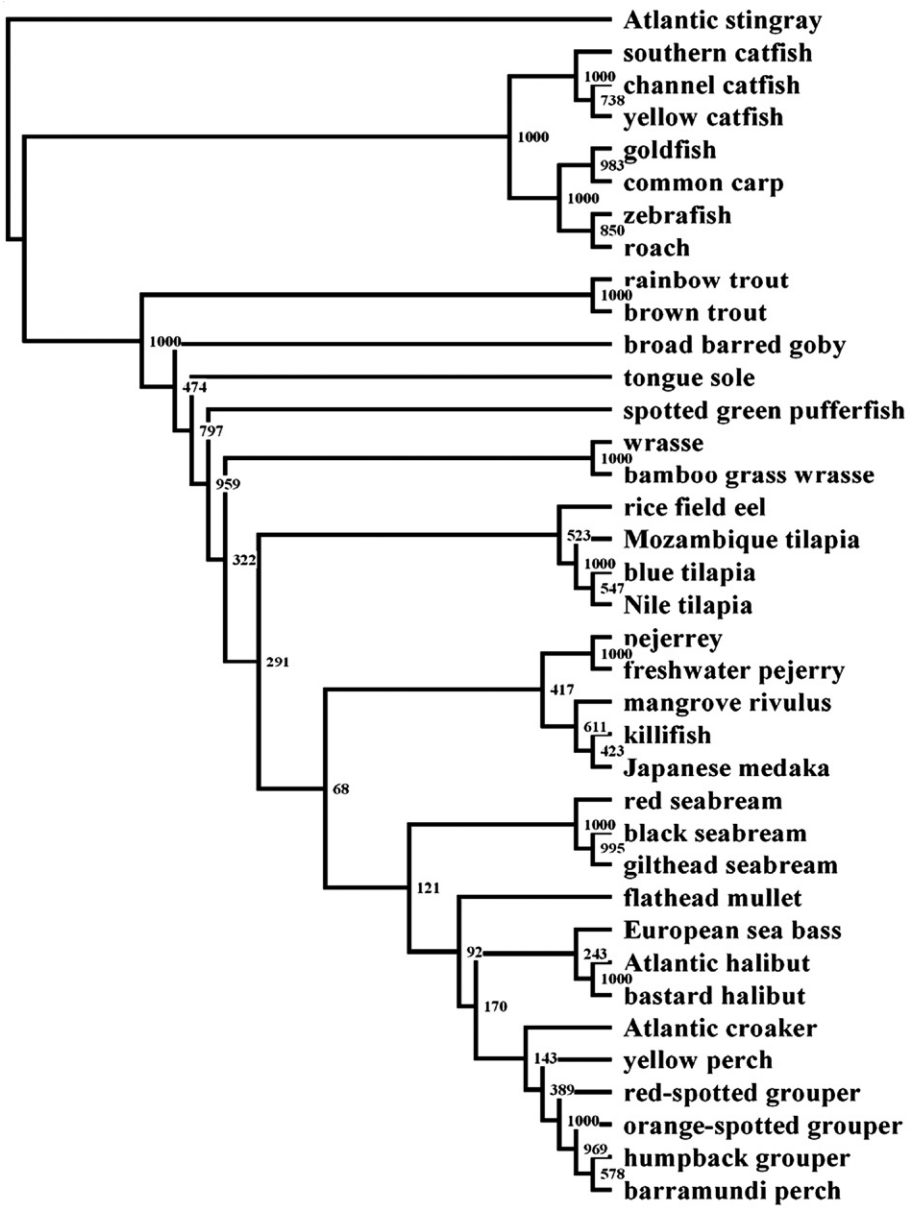


Fig. 9. Phylogenetic tree of *cyp19a1a* amino acid sequences. Tree produced using a Clustal X (1.81) alignment and TreeView (Win 32) v. 1.6.6. The tree was rooted with Atlantic stingray (*Dasyatis sabina*) aromatase (Accession AAF04617) as an outgroup representing the closest available species not in the group Teleostei. See Fig. 8 for EMBL/GenBank Accession numbers except brown trout (*Salmo trutta*) AAR04775.

There are several amino acid substitutions that are unique to the yellow perch *esr2a*, the most notable being Cys_{224} in the C domain where all other known fish have a Tyr residue with the exception of an Asn in largemouth bass. Analyses indicate that yellow perch *esr2a* shares the highest percent identity and has the closest phylogenetic relationship with largemouth bass (*M. salmoides*).

The presence of two *esr2* subtypes in at least 11 fish species (Fig. 6: fathead minnow and European sea bass partial ERβa sequences not included) spanning a number of families (Sparidae, Sciaenidae, Moronidae (Halm et al., 2004), Centrarchidae, Cichlidae, Fundulidae, Cyprinidae, Rivulidae) raises the distinct possibility that yellow perch (Percidae) has a third, uncharacterized, ER sequence (*esr2b*). The variability in size of the F and, particularly, A/B domains are the major contributing factors to overall size differences among different ERs. Of the 27 full length teleost *esr1* cDNA sequences available, ten of these have an extended 5′ end. These differences in A/B domain length do not seem to follow any significant evolutionary or phylogenetic pattern and the occurrence of a short and long A/B domain in two species of unrelated fish (channel catfish (*Ictalurus punctatus*) and rainbow trout indicates the potential for multiple *esr1* transcripts in other species (Patiño et al., 2000; Pakdel et al., 2000). In rainbow trout, the two variants show different tissue expression (Menuet et al., 2001) and data suggest that the additional residues in the A/B domain modify the hormone-independent *trans*-activation function of the receptor (Pakdel et al., 2000).

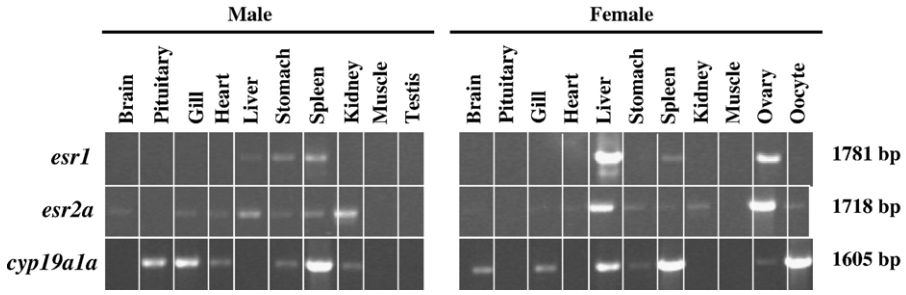


Fig. 10. Sex-specific tissue expression of *esr1*, *esr2a* and *cyp19a1a* in male and female yellow perch. For PCR primers used see Table 2 and bp sizes of products are shown to the right of the figure. Template used was 900 ng of cDNA generated from the pooled mRNA of three adult yellow perch for each sex-specific tissue. PCR products were then run on a 1% agarose gel and visualized using ethidium bromide staining. cDNA template quality was verified by analyzing for β-actin mRNA levels using real-time quantitative PCR (qPCR) (data not shown). Identity of the products was confirmed via DNA sequencing of the PCR products from several different runs.

The full length *cyp19a1a* cDNA of the yellow perch was cloned by directional RACE procedures and was shown to encode a protein of 518 amino acids. Two potential initiation ATG sites are located at 69 bp and 99 bp, however neither of these two initiation codons represents the optimal sequence for initiation as described by Kozak (1986). A similar situation has been identified in the *cyp19a1a* cDNAs in several other teleost species (Fukada et al., 1996). Most RNAs contain a polyadenylation signal of either AATAAA or ATTAAA, but in yellow perch *cyp19a1a* only a putative (AGTAAA) (Sheets et al., 1990) polyadenylation signal was found 20 bp upstream of the poly A tail. Similar putative polyadenylation signals (ATAAA) were found in Japanese medaka (*Oryzias latipes*) *cyp19a1a* (Fukada et al., 1996) and fathead minnow *cyp19a1b* (Halm et al., 2001). Yellow perch *cyp19a1a* contains four conserved regions present in all steroidogenic cytochrome *P-450*s, with the region of highest homology being the heme-binding region (Graham-Lorence et al., 1991). Several residues in the heme-binding region have been indicated as being important for the binding of the heme group (Chen and Zhou, 1992) and almost all of these are conserved throughout the known fish sequences. Two glycines in the heme-binding region, Gly_{443} and Gly_{445}, are reported to be invariant, but it should be noted that humpback grouper (*Cromileptes altivelis*) *cyp19a1a* does not exhibit this structure (Gardner et al., 2005). Phe_{446}, another reported invariant residue, brackets the Cys_{449} and, along with Lys_{452} and the human Ala_{438} (corresponding to yellow perch Val_{450}), forms a pocket for the thiolate ligand (Chen and Zhou, 1992). The human Ala_{438} is replaced with a Val residue in all known fish (including Atlantic stingray), birds and reptiles but not in any mammals or amphibians, resulting in a very curious evolutionary pattern.

In this study, female liver and ovary tissues had greater expression of *esr1* and *esr2a* than any other tissue, supporting their role as estrogen target tissues associated with reproduction. Male liver tissue showed low to moderate expression of both ERs, but testis tissue did not show expression, despite expression of all three ERs in testis of other teleosts (Choi and Habibi, 2003; Halm et al., 2004; Pinto et al., 2006). Neither male nor female yellow perch pituitary tissue showed expression of either ER despite pituitaries of other teleost species being directly responsive to estrogen *in vitro* (e.g. Elango et al., 2006). *Esr2a* showed a much more global expression than *esr1* in both males and females with noteworthy expression in brain, stomach, kidney and oocyte tissues. While the highest levels of both ERs in females were clearly seen in liver and ovary tissues, the highest *esr1* and *esr2a* expression in males occurred in spleen and kidney tissues, respectively. A likely explanation for the occurrence of *esr1* and *esr2a* expression in the male kidney is that unlike mammals, teleost fish lack a discrete adrenal gland and corticosteroidogenesis occurs in the interrenal cells which are distributed around the postcardinal vein in the anterior (head) part of the kidney (Iwama et al., 2006). The steroidogenic acute regulatory protein (StAR) protein, which is involved with steroid biosynthesis, is located in these tissues. Recently, Nunez and Evans (2007) cloned the Atlantic croaker StAR cDNA and found high levels of expression in head kidney of this teleost. The rat StAR gene promoter has an estrogen receptor half-site (Sandhoff et al., 1998) providing a possible mechanism for estrogenic modulation of corticosteroidogenesis and an explanation for ER expression in head kidney tissue of yellow perch.

Few studies have investigated sex-specific tissue expression levels of *cyp19a1a* (excluding gonads), however several tissues (brain, pituitary, heart, liver and kidney) in yellow perch showed distinct sex-specific patterns of expression. As a rare exception, a study on Atlantic halibut by van Nes et al. (2005) examined sex-specific expression in brain, gill, heart, intestine, kidney, liver, pituitary, spleen and gonad. They reported sex-specific differences in gonad and kidney expression, with female ovary and male kidney tissues showing expression. This study shows similar results in yellow perch, but while most studies showed high expression levels of *cyp19a1a* in ovary tissue this study only found low to moderate levels. It is possible that levels of *cyp19a1a* expression in ovary, oocytes and pituitary are very sensitive to seasonal and reproductive status, possibly giving some explanation to conflicting results (Halm et al., 2001; Choi et al., 2005). To highlight this, Matsuoka et al. (2006) had conflicting results to van Nes et al. (2005) on expression of *cyp19a1a* in gill, gonad, intestine, heart and liver of the same species, Atlantic halibut. They contend that, while the method of detection was different between the two studies, the life stage

of the fish (immature vs. mature, reproductive status, etc.) is likely a more significant factor.

Other studies have found high expression levels of *cyp19a1a* expression in spleen tissue (Choi et al., 2005; Luckenbach et al., 2005; van Nes et al., 2005) and even whole blood (Zhang et al., 2004), as the spleen is involved in blood cell (both red and white) production. A function of splenic *cyp19a1a* is the autocrine synthesis of estrogen releasing cytokines IL-2 and IL-6 from the splenic T lymphocytes which maintains an immune response after trauma-hemorrhage (Samy et al., 2003). This raises the possibility of an artificial spike in splenic *cyp19a1a* transcription derived from the sampling methodology. In this study, spleens were removed within 15 min of the first incision, however transcription levels have been shown to respond to a trauma within an hour. Whether *cyp19a1a* mRNA levels in spleen tissue are increased from the sampling methodology or not, the prevalence of fish species showing *cyp19a1a* expression in spleen tissue indicates a connection between immune function and steroidogenesis.

Yellow perch showed high levels of *cyp19a1a* expression in oocytes. Studies in rainbow trout (Tanaka et al., 1992) and Nile tilapia (Chang et al., 1997) have shown detectable expression in pre-vitellogenic oocytes, increasing toward mid-vitellogenesis, after which it declines sharply to non-detectable levels in post-vitellogenic oocytes. Ciereszko et al. (1997) found that yellow perch have synchronous oocyte growth during autumn and winter and by February oocytes have completed vitellogenesis. While Kolkovski and Dabrowski (1998) demonstrated that yellow perch can be manipulated to spawn out of season, they did so by completely inverting the photothermal conditions, while an earlier study indicated that yellow perch ovulation and spawning are rather recalcitrant to minor changes in photo-thermal conditions (Ciereszko et al., 1997). The yellow perch used in this study for tissue expression analyses were kept at temperatures which fluctuated slightly (1–2 °C) with season (summer higher than winter) and on a constant light:dark (14:10) cycle and spawned normally in spring (April) in prior years. Therefore, it is a plausible assumption that the oocytes taken from yellow perch in our laboratory for this study were in the post-vitellogenic stage. It remains unknown whether the results of this study can be reconciled with the studies mentioned above (Tanaka et al., 1992; Chang et al., 1997).

In regards to yellow perch SSD, Roberts et al. (2004) showed a significant increase in pituitary GH protein level in response to dietary E_2 treatment in female yellow perch. However, Jentoft et al. (2005) found no changes in serum IGF-I levels in male or female yellow perch treated with bovine GH (bGH), dietary E_2 or the combination, even though dietary E_2 treatment did promote growth. Further, Jentoft et al. (2005) found that neither recombinant yellow perch GH nor bovine bGH stimulated growth in yellow perch. Lynn and Shepherd (2007) showed high levels of GH, PRL and SL mRNA expression in both male and female pituitary tissue while this study shows little to no expression of either *esr1* or *esr2a* mRNA expression in the exact same pituitary tissue samples. This suggests that while E_2 increases GH protein levels in the pituitary of yellow perch (Roberts et al., 2004), the growth promoting actions of E_2 may work independently of GH and/or IGF-I.

In conclusion, this study provides the full length nucleotide sequences of *esr1*, *esr2a* and *cyp19a1a* cDNAs and examines their sex-specific tissue expression in yellow perch. Both ERs had the highest expression levels in female estrogen-sensitive tissues (liver and ovary) and *cyp19a1a* had a very global, albeit sex-specific, tissue expression pattern. The production and publication of these yellow perch sequences, along with those presented in Lynn and Shepherd (2007), provide molecular tools to investigate estrogen-stimulated SSD and other estrogen actions in yellow perch.

Acknowledgements

This work was funded, in part, by National Oceanic and Atmospheric Administration National Estuarine Research Reserve Graduate Student Fellowship #NA16OR2400 to S.G. Lynn. This study was also funded, in part, by grants from the National Research Initiative Competitive Grants Program/ USDA award no. 2002-35206-11629 and 2004-05124 and the support of the U.S. Geological Survey and Kentucky Water Resources Research Institute Grant Agreement No. 01HQGR0133 to B.S. Shepherd and ARS/USDA CRIS #3655-31000-020-00D. C.P. Bennett, proprietor of Panuzzo's Fish House, Lorain, OH, generously offered space and fish for the collection of tissues. J.H. Niedzwiecki, University of Cincinnati, helped considerably with the generation and interpretation of the phylogenetic trees. The views contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the U.S. Government. Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable. This manuscript is submitted for publication with the understanding that the United States Government is authorized to reproduce and distribute reprints for governmental purposes.

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